New treatment modalities in superficial bladder cancer

PhD thesis

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Introduction

In Hungary approximately 2,600 new cases of bladder cancer are diagnosed every year making it the fifth commonest cancer in men. Approximately 70% of these patients initially present with superficial bladder cancer (SBC). The standard treatment for patients with SBC is transurethral resection of the bladder tumour (TURBT), followed by adjuvant intravesical instillations with chemotherapy and/or immunotherapy (BCG). The probabilities of recurrence and progression in non muscle invasive bladder cancer at 5 years after standard treatment range from 31% to 78%. These rates illustrate the modest success of currently available treatments and underline the need for improved therapies.

Oncolytic herpes simplex virus (HSV) vectors have shown promising efficacy against a wide variety of malignancies both *in vitro* and *in vivo* and clinical trials for patients with metastatic colorectal, head and neck, breast, and prostate cancer, melanoma, and glioma have been completed. Oncovex$^{GALV/CD}$ is a third generation oncolytic herpes simplex virus 1 (HSV-1) that combines oncolysis with the expression of a highly potent pro-drug activating gene (yeast cytosine deaminase/uracil phospho-ribosyltransferase fusion) and the fusogenic glycoprotein from gibbon ape leukemia virus (GALV). Oncovex$^{GALV/CD}$ virus also contains other mutations to increase its efficacy. ICP34.5 region deletion results in tumour selective viral replication, in addition deletion of ICP47 region increases the antitumor immune response. Previous studies with Oncovex$^{GALV/CD}$ have shown enhanced cell killing and tumour shrinkage (*in vitro* and *in vivo)* within tumours derived from head and neck, colon, pancreas, lung and glioma tissue.

Bladder cancer is potentially an ideal tumour model for novel therapies because intravesical delivery is able to expose the tumour to high concentrations of virus. In addition, the umbrella cell layer of the bladder (the luminal surface of the urothelium) is not rapidly dividing and should therefore be resistant to infection and lysis by replication-competent oncolytic viruses, which selectively infect and replicate within rapidly dividing cells.

Objectives

- Test the efficacy of Oncovex$^{GALV/CD}$ *in vitro* on several transitional cell human bladder tumour cell lines (EJ, RT112, T24, VMCUB-I, TCCSUP-G, 5637, KU19-19).
- Elucidate whether expression of fusogenic glycoprotein (GALV) from Oncovex$^{GALV/CD}$ virus increases cytotoxic cell killing within these cells *in vitro*.
- Test *in vitro* the efficacy of Oncovex$^{GALV/CD}$ in the presence of 5-fluorocytosine on these human bladder tumour cell lines.
- Test *in vitro* the efficacy of Oncovex$^{GALV/CD}$ in combination with conventional chemotherapies on human bladder tumour cell lines (EJ, T24, TCCSUP-G, KU19-19).
- Set up a stable and reproducible rat orthotopic bladder tumour model that is suitable to evaluate the effectivity of different therapeutical options *in vivo.*
- Test the *in vitro* the efficacy of Oncovex$^{GALV/CD}$ on the rat bladder tumour cell line (AY-27) with the fusion and prodrug assay
- Assess the effectiveness of QRT-PCR *in vivo* in detecting tumour growth using urine and tissue samples on the rat bladder tumour model.
- Evaluate the effectivity of bioluminescence imaging technique in detecting tumour growth *in vivo* on the rat orthotopic bladder tumour model.
- Evaluate *in vivo* the efficacy of Oncovex$^{GALV/CD}$ on our previously developed rat orthotopic bladder tumour model

Materials and methods

Oncovex$^{GALV/CD}$ and Oncovex^{GFP} (as control backbone) virus were used in the study, which were previously described by Simpson et al. Human bladder transitional cell cancer (TCC) cells (EJ, T24, RT112, VMCUB-I, TCCSUP-G, 5637, KU19-19) and a rat bladder cancer cell line (AY-27) were studied.

To study the effectivity of the fusogenic gene human TCC cells were infected with Oncovex$^{GALV/CD}$ or Oncovex^{GFP} at different MOIs and incubated for 48 hours. Cells were then either fixed with glutaraldehyde and stained with Crystal Violet, or treated with MTS reagent and measured by densitometer.

To study the effectivity of the prodrug activating gene human TCC cells were infected with Oncovex$^{GALV/CD}$ or Oncovex^{GFP}. After 30 minutes the virus was removed, and media containing different concentrations of 5-FC was added. After 48 hours incubation the cell supernatant was centrifuged and heat inactivated at 60°C. The resulting supernatants were added to fresh test cells and incubated for 48 hours. Cells were then either fixed with glutaraldehyde and stained with Crystal Violet, or treated with MTS reagent and measured by densitometer.

The effect of combination of Oncovex$^{GALV/CD}$ and chemotherapeutic agents on cell proliferation was assessed by calculating combination index (CI) values. Derived from the median-effect principal of Chou and Talalay the CI provides a quantitative measure of the degree of interaction between two agents. A CI of 1 denotes an additive interaction, >1 antagonism, and <1 synergy. Experiments were done as described for *in vitro* survival assay using 4, 2, 1, 0.5, and 0.25 times the calculated ED_{50} of each agent (Oncovex$^{GALV/CD}$ and chemotherapy) in a constant ratio checkerboard design. After 48 hours incubation cells were treated with MTS reagent and measured by densitometer. CI values were calculated using CalcuSyn software.

To set up the *in vivo* rat orthotopic bladder tumour model Fischer F344 female rats were used. The animals were placed in a supine position and were anesthetised with Isoflurane. The catheter (18-gauge BD Venflon) was inserted into the bladder via the urethra. To facilitate the tumour seeding the bladder mucosa was damaged by instillation 0.1 N

hydrochloric acid followed by a rinse with 0.1N sodium hydroxide for neutralization. The bladder was washed five times with PBS and AY-27 HVEM cells (1.5-2.5x10^6 cells) were then instilled and maintained in the bladder for 1 hour. After 1 hour the catheters were removed, and the rats were allowed to void spontaneously.

To detect tumour growth urine samples were collected by holding the rats in a metabolization cage for 1 hour on day 0,4,7,11,14 after tumour implantation. On the collected urine samples and on day 28 removed bladder tissues QRT-PCR (quantitative reverse transcription polymerase chain reaction) was performed.

To detect tumour growth by bioluminescent imaging technique 1x10^6 AY-27 HL-S cells (encoding luciferase enzyme) were injected subcutaneously to the flank of the rats. In regular timepoints D-Luciferin Firefly Potassium salt (150 mg/kg) was administered and imaging was performed by the non-invasive IVIS bioluminescence imaging camera to detect luciferase enzyme activity. Results were analyzed using the Xenogen software, that provides visual photographic images of bioluminescence detection.

To evaluate the effectivity of Oncovex$^{GALV/CD}$ *in vivo* the animals were assigned into three treated groups after tumour implantation (day 0): either OncoVex$^{GALV/CD}$+5-FC **(N°=10)**, OncoVex$^{GALV/CD}$ +PBS **(N°=10)** or PBS+5-FC (control group) **(N°=8)**. Intravesical treatment of implanted tumours was carried out with OncoVex$^{GALV/CD}$ on days 7, 14 and 21 and with 5-FC on days 8, 9, 15, 16, 22 and 24 in the same manner. The bladders were removed on day 28 and assessed for tumour abundance by macroscopic and pathological evaluation.

Results

Human bladder TCC cell lines are sensitive to viral HSV oncolysis, which is enhanced by the expression of GALV glycoprotein.

A panel of 7 TCC cell lines were tested for viral HSV oncolysis. High viral replication of the oncolytic HSV (Oncovex$^{GALV/CD}$) was observed in all 7 TCC cell lines. This HSV viral replication led to a strong tumour cytotoxicity effect which was detected by MTS assay at an MOI as low as 0.001.

The expression of GALV glycoproteins enhanced this viral tumour selective killing in four out of the seven TCC cell lines (EJ, T24, VMCUB-I and 5637 cells) infected with Oncovex$^{GALV/CD}$. GALV expression led to the formation of multinucleated syncytia which were then surrounded with cells showing the more classic HSV-1-mediated effect. *In vitro* MTS assays were carried out where the formation of multinucleated syncytia increased the cytopathic effect of Oncovex$^{GALV/CD}$ when compared to the control virus. Lower levels of MTS activity were seen with OncoVEX$^{GALV/CD}$ on infected EJ (42-54% decrease in cell survival, P<0.000), T24 (35-45%, P<0.000), VMCUB-I (36-37%, P<0.000) and 5637 (35% P<0.000) cells. This suggests that the presence of GALV gene increased tumour cell killing.

Cytosine deaminase (CD)/uracil phospho-ribosyltransferase expression converts 5-FC to 5-FU metabolites that show active chemotherapeutic effect within human bladder TCC cell lines *in vitro*.

Cytosine deaminase (CD)/uracil phospho-ribosyltransferase fusion metabolizes 5-FC more efficiently than either gene alone. The cell killing effects of 5-FC metabolites were studied on 7 human TCC cells with Oncovex$^{GALV/CD}$ or Oncovex^{GFP} in the presence or absence of 5-FC. In EJ cells infected with Oncovex^{GFP} no cell death was seen with or without 5-FC, whereas in the presence of both Oncovex$^{GALV/CD}$ and 5-FC effective cell killing was seen. Results were similar in a range of human bladder tumour cell lines, including RT112 cells, TCCSUP-G cells, 5637 cells, KU19-19 cells. EJ cells showed 78% (P <0.000), RT112 and KU19-19 cells showed 70% (P <0.000), TCCSUP-G and 5637 cells showed 53% (P<0.000) decrease in tumour cell survival on *in vitro* MTS assay. From these results we concluded that five out of

seven TCC cells were sensitive to metabolites of 5-FC after infection with Oncovex$^{GALV/CD}$ (in the presence of 5-FC).

Oncovex$^{GALV/CD}$ and chemotherapeutic agent mitomycin C, show synergistic interaction on bladder TCC tumour cell lines, whereas coadministration with cisplatin or gemcitabine is antagonistic.

From the currently used chemotherapeutic agents mitomycin C (MMC), cisplatin and gemcitabine were studied in combination with Oncovex$^{GALV/CD}$. (We tested TCC cells including EJ, T24, TCCSUP-G and KU19-19.) We observed synergistic cell killing with Oncovex$^{GALV/CD}$ and MMC on EJ (ED_{50} 0.77 +/- 0.05), T24 (ED_{50} 0.65 +/- 0.07) and KU19-19 (ED_{50} 0.78 +/- 0.01) TCC cells. However a combination of Oncovex$^{GALV/CD}$ and cisplatin or gemcitabine was antagonistic on EJ, T24 and TCCSUP-G cells.

***In vivo* rat orthotopic bladder tumour model was set up and Oncovex$^{GALV/CD}$ treatment with or without 5-FC was studied on the model.**

To model *in vivo* human superficial bladder cancer a rat orthotopic bladder tumour model was used which was previously described by Xiao et al. AY-27 rat bladder transitional cell carcinoma cells that were previously tested for susceptibility for HSV replication were used in this model. AY-27 cells were stably transfected with the herpesvirus entry receptor (HVEM) and a clone was selected that supported infection with HSV.

In vitro fusion assay (for testing GALV gene) results showed a reduction in tumour cell survival up to 30% in the new AY-27 HVEM cell clone when infected with Oncovex$^{GALV/CD}$ compared to the Oncovex^{GFP} control. AY-27 HVEM cells were further tested *in vitro* in our prodrug assay, which showed that Oncovex$^{GALV/CD}$ can metabolize 5-FC within these cells, resulting in a decrease in tumour cell survival up to 81% when compared to controls.

In the rat orthotopic bladder tumour model to facilitate tumour seeding, the bladder mucosa was conditioned with an acid rinse followed by neutralization with alkali and then AY-27 HVEM cells were implanted. After tumour seeding a high success rate of implantation was seen (>95%). The tumour implantation procedure was well tolerated by the animals and it was also reproducible.

We planed to use QRT-PCR to demonstrate the presence of the HVEM receptor of the AY-27 rat bladder tumour cells as a marker for tumour

load *in vivo*. However while we obtained positive signals on bladder tissue QRT-PCR, signals using urine samples were insignificant, therefore tumour growth was detectable by QRT-PCR only on bladder tissue samples.

Non-invasive IVIS bioluminescence imaging camera was also not suitable to detect tumour growth *in vivo* in this rat orthotopic bladder tumour model, due to host immune response to the luciferase expression.

To study the efficacy of Oncovex$^{GALV/CD}$ *in vivo* the tumour bearing animals were assigned into three treated groups after tumour implantation: either Oncovex$^{GALV/CD}$+5-FC, Oncovex$^{GALV/CD}$ +PBS or PBS+5-FC. The results showed an 84.5% reduction in average tumour volume in the presence of both Oncovex$^{GALV/CD}$ and prodrug when compared to control (**P=0.001**) or Oncovex$^{GALV/CD}$ virus alone (**P=0.034**). A smaller amount of tumour shrinkage seen with Oncovex$^{GALV/CD}$ virus alone was not statistically significant when compared to control animals (46.4% tumour reduction) (**P=0.13**). The results were similar when comparing total bladder weights. On average the animals treated with Oncovex$^{GALV/CD}$ +5-FC were 11.5g heavier than controls, suggesting that they were in a healthy condition compared to controls.

Conclusion

- The transduction of human bladder tumour cells with viral fusogenic membrane glycoprotein (GALV) caused fusion and increased tumour cell killing *in vitro* using the OncoVEX$^{GALV/CD}$ virus.
- The transduction of human bladder tumour cells with prodrug activating (yeast cytosine deaminase/ uracil phospho-ribosyltransferase) gene promoted active metabolism of 5-FC into 5-FU and enhanced tumour cell killing *in vitro* using the OncoVEX$^{GALV/CD}$ virus.
- All human bladder tumour cell lines tested are susceptible to HSV oncolysis and showed enhanced tumour cell killing in at least one type (fusion or prodrug) of the assays when infected with OncoVEX$^{GALV/CD}$ virus.
- The combination of oncolytic transduction of bladder tumour cells with viral fusogenic membrane glycoprotein (GALV) and a prodrug activating system (CD) can further increase tumour control *in vitro.*
- The coadministration of OncoVex$^{GALV/CD}$ and mitomycin showed synergistic effect *in vitro* on human bladder tumour cells.
- The coadministration of OncoVex$^{GALV/CD}$ with cisplatin or gemcitabine showed antagonistic effect *in vitro* on human bladder tumour cells.
- AY-27 rat bladder tumour cells are rare exception in failing to support HSV entry and/or replication.
- The transfected AY-27 HVEM cell line that contains the herpesvirus entry receptor supports HSV entry and replication.
- The transduction of AY-27 HVEM cell line with viral fusogenic membrane glycoprotein (GALV) caused fusion and increased tumour cell killing *in vitro* using the OncoVEX$^{GALV/CD}$ virus.
- The transduction of AY-27 HVEM cell line with prodrug activating (CD) gene in the presence of 5-FC led to enhanced tumour control *in vitro* using the OncoVEX$^{GALV/CD}$ virus.
- The rat orthotopic bladder tumour model using AY-27 HVEM cell line was a stable and useful model that is suitable for further *in vivo* testing.

The percentage of tumour implantation using the orthotopic tumour model was strong (almost 95% after necropsy).

- QRT-PCR on the removed bladder tissue samples (after necropsy) showed effective detection of tumour growth, but failed to detect a signal in urine from the same animals *in vivo*.
- Non-invasive IVIS bioluminescence imaging camera was also not suitable to detect tumour growth in this rat orthotopic bladder tumour model.
- OncoVex$^{GALV/CD}$ intravesical therapy with the combined transduction of viral fusogenic membrane glycoprotein (GALV) and a prodrug activating system (CD) in the presence of 5-FC prodrug led to enchanced local tumour control within the bladder *in vivo* in the rat orthotopic bladder tumour model.

List of publications

Publications related to the thesis:

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